USDA COOPERATIVE AGREEMENT

Reduction of Potential Health-Related Tobacco Constituents by Molecular Genetics

Final Report

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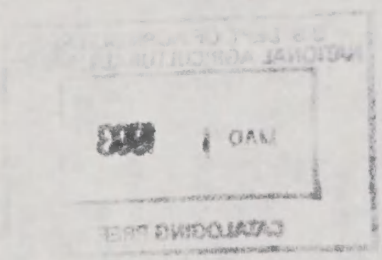

Work Results

The purpose of this research was to understand and ultimately reduce the levels of the potentially health-hazardous polyunsaturated fatty acids (which are major constituents of tobacco leaves) and polyene compounds such as carotenoids and chlorophylls which can be converted into procarcinogenic polycyclic aromatic hydrocarbons upon pyrrolysis. This can be accomplished by promoting their conversion into non-hazardous, volatile peroxidation products. A class of enzymes which can do this are known as fatty acid oxidases, carotene oxidases or lipoxygenases. This project was conducted in three phases: 1. Characterization of the volatile compounds produced by tobacco leaves and studies of their biogenesis; 2. Perfection of gene transfer systems for Burley tobacco and 3. Biochemical and molecular characterization of lipoxygenases.

All of the major volatiles produced by a number of tobacco genotypes (including Burley tobacco, KY 14) were seperated and identified. Inhibitor studies and structural analysis indicates that a number of the major volatiles are lipoxygenase products. Surprisingly, it appears that most of the volatiles produced by tobacco leaves are not derived from the trichomes. It appears rather than they are derived from another part of the leaves. The vast bulk of the volatiles produced by damaged, diseased or wounded tobacco leaves are lipoxygenase products.

A major components of tobacco leaves are the membrane and other glycerolipids and more than 60% of the fatty acids of lipids are the polyunsaturated fatty acids linoleic (18:2) and linolenic (18:3) acids (Table 1) which can be converted by lipoxygenase into non-hazardous products.

Table 1. Fatty acid composition of leaf lipids of two Burley tobacco cultivars.

Fatty acid	KX-14	KY-17
16:0	31.6	29.9
16:1	1.6	1.7
18:1	2.9	3.9
18:2	39.6	40.8
18:3	24.2	23.7

Analysis of the volatile compounds produced from tobacco leaves. The volatile compounds from fully expanded whole leaves of 4 greenhouse-grown vegetative (green) tobacco lines were isolated by entrainment in purified air followed by Tenax

trapping, characterized by capillary GC and GC-MS and quantified by capillary GC. Identified headspace compounds, which comprised about 50% of the number of GC peaks and weight of the total estimated volatiles, were: E-B-ocimene, linalool, Z-3-hexenyl acetate, Z-3-hexen-1-01, B-caryophyllene, E-B-farnesene, solonone, methyl salicylate, nicotine and neophytadiene. Yields of total estimated volatile compounds among the replicated leaf samples of the tobacco lines ranged from 6.6-38.5 ng/g net weight (Table 2). The means of amounts of volatile components that are believed to be emitted as a result of lipoxygenase activity were performed with 5-L enrainment flasks than with 12-L flasks. The leaf surface emissions of individual volatile compounds were examined using lines with different leaf trichome morphologies: KY14 (commercial Burley), Ti 1068 (having twice the normal trichome density), Ti 1406 (having non-secreting trichomes) and Ti 1112 (having simple [headless] trichomes). Surprisingly, the line with headless trichomes (Ti ~~1068~~ 1112) had the lowest total yield of volatiles. The line with the greatest number and density of glandular headed, secreting trichomes (Ti 1068) had the lowest total yield of volatiles. The type with glandular headed, non-secreting trichomes was second highest and KY14 second lowest in volatile compound yield. Model system studies and use of LOX inhibitors indicates that the volatile compounds derived from polyunsaturated fatty acids are LOX products. Many of the other volatile compounds which are oxidation products are also thought to be derived via LOX action particularly those derived from carotenoids. Quantitative estimates of headspace total volatile yields are given in Andersen et el (1986). These findings represent preliminary information obtained from a survey of

Table 2. Yields of Total Headspace Volatiles from Different Vegetative Plant Materials.

Plant material description	Entrainment gas	Sample weight, kg	Yield ng/g
tobacco (KY 14 burley): stalk, 7 weeks growth in greenhouse	air	1	10
stalk, 14 weeks growth in greenhouse	air	1	71
leaf, 13 weeks growth in greenhouse	air	1	112
leaf, 14 weeks growth in greenhouse	air	0.1	980

several factors investigated for their effects on yields of headspace volatiles. The factors were plant age, plant part, plant genetics (genus, species, variety), growth conditions, entrainment gas and weight of sample. Yields from tobacco leaf were greater than from tobacco stalk. The order of increasing amounts of volatiles among leaves of the 3 plant

species was tobacco, wheat, strawberry. Nitrogen and air were both effective for entrainment of volatiles, and, in the case of wheat, the yields of total volatiles did not differ by more than 25%. There was a varietal difference in the yields of volatiles from strawberry plants. These tentative results will provide guidelines for further related studies.

Fatty acids as precursors of volatiles in steam distillate-hexane extracts of tobacco and wheat. Relative abundances of specific fatty acids in hydrolysates of 2 varieties of burley tobacco stalk are given Andersen et al. (1986) and for leaves in Table 1. There were relatively small varietal differences in content between the 2 tobacco stalk materials, but there were rather large differences between tobacco stalk and wheat. Tobacco contained more palmitic, oleic and linoleic acid than wheat, whereas wheat contained more palmitoleic and linolenic acid than tobacco. Evidence that supports a direct precursor-product relationship between a fatty acid and a specific steam distilled volatile compound is found in our results that show hexanal and trans 2-nonenal (which presumably originate from their linoleic acid precursor) account for a larger proportion of the total oil from steam distillates of tobacco stalk than is the case for wheat plants (Andersen et al, 1986). On the other hand, trans 2-hexenal and the sum of nonadien-aldehydes and alcohols, which presumably originate from linolenic acid, account for a smaller proportion of the oil of tobacco stalk than of wheat.

Several studies showed that chemical agents can inhibit lipoxygenase and cycloxygenase-mediated changes with fatty acid substrates. For example, it has been determined that lipoxygenase-catalyzed oxidation of linoleic acid was inhibited by various antioxidants such as catechol, nordihydroguaiaretic acid and quercetin. Recently, it has been shown that formation of carbonyl-containing compounds and alcohols that characterize the fresh fish aroma of emerald shiners was inhibited when fish were sacrificed and immediately treated with solutions of acetylsalicylic acid or stannous chloride which are inhibitors of cyclooxygenase and lipoxygenase, respectively. It has also been shown that phenidone was potent inhibitor of lipoxygenase and cyclooxygenase in vitro (Andersen et al, 1986).

Studies were carried out with tobacco and wheat to determine the effects of known inhibitors of lipoxygenase and cyclooxygenase on the yields of volatile oils in steam distillate-hexane extracts. Although there is considerable evidence that lipoxygenase is important in higher plant metabolism, it is not certain whether cyclooxygenase plays a similar role. In the case of volatiles from tobacco stalk sections, each agent used did not decrease the yields. Rather, increases were observed which may have resulted from the higher ionic strength of the solutions. It was decided to try other specific agents using homogenized plant tissue for

the steam distillations. We reasoned that homogenization would release more native enzymes and would allow maximum exposure of endogenous plant substrates to the inhibitors. Wheat was used because it could be easily homogenized in contrast to tobacco stalk.

Phenidone (1-phenyl-3-pyrazolidone) inhibited total volatile oil yield in wheat 43%, which was the largest decrease we found (Andersen et al, 1986). This agent has been used as a developer in the photographic industry. Nordihydroguairetic acid, an aromatic polyphenol caused a 32% inhibition in total volatile oil yield. Acetylsalicylic acid, on the other hand, did not affect yields. On the basis of these results and the known target enzymes inhibited by these agents, the lipoxygenase system appears to play a significant role in the production of volatile compounds in plants. Comparisons of paired phenidone-treated and control yields at a given Kovats index indicate relatively strong reductions of 6-carbon volatiles with phenidone. Compounds that were reduced by phenidone include hexanal, 2-hexenal, 2-hexen-1-ol and β-ionone. On the other hand, there were some increases of 9-carbon compounds with phenidone treatment.

Studies were performed to isolate, identify, and quantify headspace volatiles of vegetative (green) burley tobacco leaf. A comparison was made of the quantities of volatile compounds obtained form genetic lines of burley tobacco that varied in trichome morphology and density to determine whether the majority of leaf volatiles are emitted from the glandular heads of secreting trichomes (Andersen et al, 1988).

These lines were kindly provided by the ADODRS.

Quantitative Estimates of Total Headspace Volatiles. The means of amounts (wet-weight basis) of total volatile compounds (identified and unknown) in the 5-L run A trapped headspace preparations (Andersen et al, 1988) from fully expanded leaves for each of the four tobacco lines were as follows: TI 1112, 38.5 ng/g; TI 1406, 25.0 ng/g; TI 1068, 16.6 ng/g; KY 14, 15.7 ng/g. Corresponding amounts in the 12-L run B preparations were as follows: TI 1112, 30.0 ng/g TI 1406, 12.6 ng/g; TI 1068, 8.7 ng/g; KY 14, 6.6 ng/g. The purpose for using two different sized entrainment vessels for headspace analyses was twofold: (1) to demonstrate the effect of small partially controlled amounts of leaf wounding on the yields of total and individual headspace volatiles; (2) to determine the compositional nature of lipoxygenase-generated volatiles.

The mean yields of total volatiles among the four tobacco lines obtained with 5-L entrainment flasks were 1.3-2.4-fold higher than the corresponding yields obtained with 12-L flasks. One probable explanation for this increase is the increased injury of leaves that occurred when they were placed

in the narrower opening of the smaller flask (45/50$\bar{S}$) as compared to the larger flask (71/60$\bar{S}$). Such injury of plant tissue as, for example, detachment of leaves, leaf tearing, fruit disruption, etc., is known to activate a group of enzymes including lipoxygenase and hydroperoxide lyase required for the generation of volatile compounds such as aldehydes, alcohols, and esters containing six and nine carbon atoms. A second and perhaps less tenable explanation for more total volatiles obtained with smaller flask size is that there was a greater linear air flow per unit area of leaf surface and less chance of binding to active sites on the glass in the smaller flask. Thus, more volatiles may have been entrained in the smaller flask even though a uniform flow rate was maintained in the Tenax traps connected to both flasks. Comparison of our observed yields of volatiles in leaves (and stems) of other species such as clover (100 ng/g) and wheat (10-50 ng/g) indicates that tobacco leaves grown under our conditions emitted gross quantities of volatiles similar to the other compared plant species.

The results indicate that the leaves from tobacco lines with nonsecreting and fewer trichomes per unit area (TI 1406) and with headless nonsecreting trichomes (TI 1112) emitted higher total yields of headspace volatiles than leaves from lines with glandular-headed secreting trichomes of higher density (TI 1068, KY 14). The data are consistent with emissions of large fractions of the total volatile compounds from the epidermal cells or stomata of the leaf surfaces, rather than for glandular secreting trichomes, per se.

Identification of Tobacco Leaf Headspace Volatiles. The identification of 10 volatile compounds from tobacco leaves estimated to comprise about 50% of the weight of total estimated volatiles emitted from the 4 tobacco lines are summarized in Andersen et al (1988). Each compound represented a single GC peak, and their sums accounted for about half the total number of GC peaks obtained for each tobacco line. All the identifications except for that of linalool were based on their presence in a headspace isolate of KY 14 tobacco leaves that was more concentrated (X4) than that used for volatile quantifications. Linalool was identified in a headspace isolate of TI 1068 leaves.

Quantiative Estimates of Individual Headspace Volatiles. Comparisons of amounts of a compound (means on a wet-weight basis) obtained from headspace isolates of TI 1112, TI 1406, TI 1068, and KY 14 obtained with a 5-L entrainment vessel (run A) with those of a 12-L vessel (run B) indicated that significant differences were obtained between runs A and B only for (Z0-3-hexenyl acetate and (Z)-3-hexen-1-ol, the only two components identified that are considered to be products of lipoxgyenase-lyase activity. The elevated yields of these two known products of lipoxygenase-lyase activity in run A

compared to run B account for part of the higher yields of total volatile compounds in run A.

Amounts of individual components of the headspace volatiles from leaves of each tobacco line obtained during analyses performed with the 12-L entrainment flask are given in Andersen et al (1988). The results obtained do not support the view that the exudate-secreting glandular heads of leaf trichomes emit the majority of headspace volatiles. However, there were some significant differences in yields of some compounds among the genetic lines of tobacco. It is interesting to note that leaves of the TI 1112 and TI 1406 lines with headless and nonsecreting glandular-headed trichomes respectively, emitted greater quantities of identifiable volatiles than leaves of the TI 1068 and KY 14 cultivars that had secreting glandular-headed trichomes.

Quantities of the monoterpenoid (E)-β-ocimene were larger from the tobacco line with headless nonsecreting trichomes (TI 1112) than they were from tobacco lines with nonsecreting glandular headed trichomes (TI 1406) and "normal" glandular headed secreting trichomes (TI 1068, KY 14). In contrast to the monoterpenoid (E)-β-ocimene, significant quantities of the sesquiterpenoid β-caryophyllene accumulated only in the leaf headspace of the "normal" glandular-headed trichome tobacco line, namely TI 1068. β-Caryophyllene was reported present in tobacco and in the headspace of green tobacco stalk (Andersen et al., 1986 and 1988).

The acrylic diterpenoid neophytadiene was generally the most abundant compound in the leaf headspace of the tobacco lines. The compound has been found in most tobacco types and was present in the headspace of green tobacco stalk (Andersen et al., 1986). However, there was significantly less neophytadiene from TI 1068 leaves than from TI 1406 leaves.

Solanone was present in the headspace of TI 1112 leaf but was not detectable in the headspace of green leaves of the other tobacco lines at the concentrations used for quantifications. Solanone was previously reported in the steam distillate of green tobacco stalk (Andersen et al., 1986) and in the essence and essential oil of flue-cured tobacco.

Methyl salicylate was quantified in the headspace of TI 1112 and TI 1406 tobacco lines, but it was not detectable at concentrations used for quantifications in TI 1068 or KY 14 headspaces. The presence of this compound was recently reported in the headspace of green tobacco stalk (Andersen et al., 1986).

Quantities of (Z)-3-hexenyl acetate were present at detectable concentrations in the headspace of TI 1112 and TI 1406 leaves. (Z)-3-Hexen-1-ol commonly referred to as leaf

alcohol was the second most abundant volatile compound, and it was emitted by all of the tobacco leaf samples (Z)-3-Hexenyl acetate and (Z)-3-hexen-1-ol were not detected in the headspace of green tobacco stalk (Andersen et al., 1986). Lipoxygenase-lyase-mediated products in the headspace of plant leaves such as tobacco can be regarded as inducible, naturally occurring, volatile compounds that may have a physiological or defense role that has not been elucidated. The theory that the major C_9 volatile products are derived via the enzyme lipoxygenase was supported by studies that utalized on inhibitor of lipoxygenase Hamilton-Kemp et al (1987). The production of many of the volatile compounds was shown to be heat labile.

Gene Transfer System for Tobacco.

This project assisted in the development of a versatile and effective vector construct system for the expression of foreign genes in tobacco (Schardl et al, 1987). We have developed the following transformation system for Burley tobacco based on the leaf disc system.

Agrobacterium tumefaciens: Overnight cultures of pGV3850 + chimeric integrate are grown in 30ml of YEP broth at 28xC in the presence of Rif^{100} plus Spc^{100} and sr^{100} in the case of pMON237. The Agrobacterium culture is washed at maximum speed on the IEC centrifuge for 15 minutes to pellet the bacteria and resuspended in 20 to 30ml of YEP broth. Alternatively, an ultra centrifuge at (Beckman using a JS 13.1 at 10,000 rpm for 10 min will pellet the bacteria as desired. The exact titer of the bacteria appears to be unimportant for efficient tranformation.

Tobacco:

1. Sterlize leaves as listed below or use aseptically grown plants. If aseptic plants are used a brief EtOH rinse may be necessary to allow the liquid inoculum to infiltrate into the leaf disc.

   A. 30 seconds 70% Etoh
   B. 15 minutes 10% chlorox w/Liquinox
   C. 2 sterile water rinses

2. Use 6mm diameter paper hole punch to produce leaf discs, avoid midribs and veins. Young leaves appear to give more uniform results. Recently we have switched to using 5mm discs. The smaller discs remain in more intimate contact with the media.

3. Submerge leaf disc in overnight culture of Agrobacterium tumefaciens. Do not over saturate the leaf disc.

4. Gently blot the disc to remove any excess liquid using the center transfer disc. The blotting can be omitted as it does

not appear to be necessary and does not reduce the bacterial infection.

5. Incubate 4 inoculated leaf discs/plate on DBI as indicated below.

> The Feeder layer plus double filter paper layer: Typically we will wash 50 ml of Su/Su suspension cell at a setting of 50 on the IEC centrifuge for 10 minutes. This results in a packed cell volume of liquid DBI. DBI is used to ensure that the suspension cells used as a feeder culture are suspended in a medium with the same hormone constitution as the regeneration plates. The suspension culture density is at a proper density so that 1.5ml of culture will produce a thin layer of cells over the entire surface of a Lab-tek 60 x 20mm (#4036) Petri dish.

6. Recently we have noticed that the feeder layer is not necessary for efficient transformation and its use, except in certain circumstances, has been eliminated.

7. After 2 to 4 days the leaf discs are transferred to selection media consisting of 200ug/ml of mefoxin (cefoxtin) antibiotic to eliminate the bacteria plus 300 ug/ml kanamycin to select for transformed cells. Claforan (cefotaxime) can be used in the place of mefoxin. Claforan does not have the cytokini-like effect that mefoxin has and may be useful during rooting selection.

8. Shoot development should be observed in two to four weeks. Excise and transfer to R1/2N for root initiation on 50 to 100ug/ml kanamycin plus claforan (100ug/ml). This second screen for Km resistance will delay rooting up to 4 weeks. To eliminate this delay shoots can be rooted in the absence of any antibiotic and then transferred to selection media. This reduced the time delay by up to 2 weeks.

9. Before transferring the rooted plantlets to soil leaf discs are taken and callusing initiated in the presence of Km at 300ug/ml. According to Fraley (personal communication) if the LD recalluses at this stage you have successful Km integration and expression. He has not found one case of a non-transformed plant that calluses in this test. In our experience greater than 95% of our regenerated plants will callus in the presence of Km and no controls (approximately 500 LD) have callused in the presence of Km.

Biochemical and Molecular Characterization of Lipoxygenases.

The three soybean lipoxygenase isozymes were purified to homogeneity and antibodies were produced. We assisted in the cloning of all 3 of the lipoxygenase cDNAs of soybean seeds (Start et al, 1986). All of these have since been fully sequenced (Hildebrand et al, 1988). Soybean lipoxygenase

genomic DNA clones have also been isolated and partially characterized (Fig. 1). One of the genomic DNA clones appears to contain sequences of both lipoxygenase 1 and 2 consistent with the tight genetic linkage found for lipoxygenase 1 and 2.

Preliminary studies indicated that tobacco DNA contained sequences homologous to soybean lipoxygenase cDNAs. Therefore, a genomic DNA library form N. tabacum cloned in lambda phage Charon 32 (kindly provided by Dr. R. Goldberg) was screened with the soybean cDNA coding for LOX-3 (pLX-10). Three clones containing putative lipoxygenase genes have been isolated from the genomic DNA library. A detailed restriction map for two of them has been established (clones C1 and L1). Charon 32 clone L1 contains 12.4 kb, clone C1 about 16.45 kb tobacco DNA as an insert (Bookjans et al (1988). The homologous DNA sequence to the cDNA probe of pLX-10 has been pinpointed in both of them. The orientation of clone C1 with respect to the 5′ and 3′ end of the putative lipoxygenase gene was determined by comparing the DNA sequence of pLX-10 (G. Bookjans, unpublished) to its homologous counterpart in clone C1. From this comparison, it was concluded that the entire putative LOX-gene of clone C1 is located on the Eco R1 restriction enzyme fragments of 7 and 0.8 kb (Bookjans et al., 1988). Subsequently it was decided to subclone both Eco R1 restriction enzyme fragments and to determine their DNA sequences. It was found that both Eco R1 fragments indeed contain the DNA sequence of a putative lipoxygenase gene. The deduced amino acid sequences of this putative LOX gene (pGBC1) is presented in Bookjans et al (1988) also contains the amino acid sequences of three soybean lipoxygenases which were deduced form the DNA sequences of the corresponding cDNA clones. The amino acid sequence of LOX-3 shown in Bookjans et al (1988). is partly encoded in pLX-10 (G. Bookjans, unpublished), a full-length cDNA clone for LOX-3 and its DNA sequence was obtained by R. Yenofsky (unpublished data). The amino acid sequence of LOX-1 was taken from Shibata et al. and kindly provided by Axelrod. The amino acid sequence deduced from pLX-65 is based on a corrected version (G. Bookjans, unpublished) of the DNA-sequence published by Start et al. (1986). Evidently the primary structures of the lipoxygenases shown in Bookjans et al. (1988) exhibit high degrees of homology. Furthermore, it should be mentioned that the putative liposygenase gene from N. tabacum also contains the same apparent intron/exon splicing sites as the gene for LOX-3 in soybeans (data not shown) indicating a high degree of conservation of the soybean and tobacco LOX genes.

We have found that the soybean seed lipoxygenase isozymes show distinctly different properties in terms of the catalysis of secondary lipid oxidation product formation. We find lipoxygenase activity in virtually all parts of tobacco plants examined. Both a high and low molecular weight lipoxygenase antibody reactive protein is seen in tobacco leaves (Hildebrand et al, 1987).

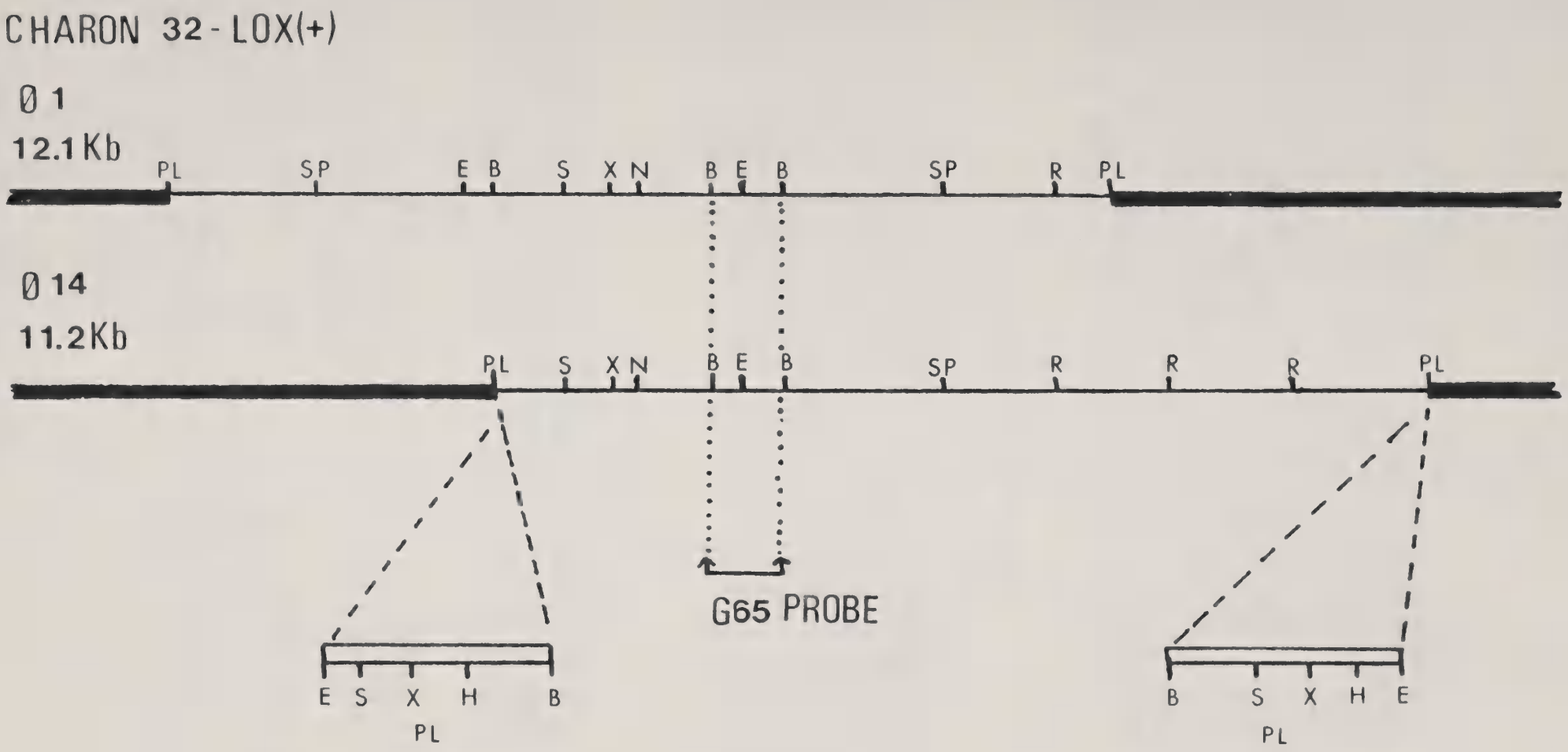
CHARON 32 - LOX(+)
Ø 1
12.1 Kb
PL
SP
E B
S
X N
B E B
SP
R
PL
Ø 14
11.2 Kb
PL
S
X N
B E B
SP
R
R
R
PL
G65 PROBE
E S X H B
PL
B S X H E
PL

Figure 1.

We have constructed plant transformation vehicles with the lipoxygenase 3 gene in the plus and minus sense direction (driven by the 35S promoter). Tobacco cells have been transformed with these constructs and transformed plants regenerated. All of the transformed plants have expressed the selectable marker (NPT II) at good levels, but none of the plants have shown high levels of expression of the inserted lipoxygenase gene. Some have shown low levels of expression of the introduced gene and are being evaluated. Additional transgenic plants will be produced with other lipoxygenase-vector constructs. Transformed tobacco plants with large alterations of particular lipoxygenase isozymes will be examined for changes in the conversion of polyunsaturated molecules into various secondary oxidation products with particular emphasis on flavor and aroma compounds.

Scientific Colaborators

In addition to the scientific collaborators listed on the cover page, two postdoctoral fellows were supported at least in part on the project: Drs. Gary Benzion and Mitchell Altschuler. Dr. Benzion went to work for Dupont Co. in Wilmington, DE. in 1987 and is now with the US patent office in Washington, DC. Dr. Altschuler did another postdoctoral for a year at Melbourne Univ. in Australia and is now an assistant professor at Northern Illinois Univ. at DeKalb, IL. Scott Wiley began a MS thesis program on this project but he could not continue due to a tragedy in his family.

Publications

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